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The PPR protein RARE1-mediated editing of chloroplast *accD* transcripts is required for fatty acid biosynthesis and heat tolerance in *Arabidopsis*

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ABSTRACT

It has been reported that *Arabidopsis* chloroplast *accD* transcripts undergo RNA editing and that loss of *accD*-C794 RNA editing does not affect plant growth under normal conditions. To date, the exact biological role of *accD*-C794 editing has remained elusive. Here, we reveal an unexpected role for *accD*-C794 editing in response to heat stress. Loss of *accD*-C794 editing results in a yellow and dwarf phenotype with decreased chloroplast gene expression under heat stress, and artificial improvement of C794-edited *accD* gene expression enhances heat tolerance in *Arabidopsis*. These data suggest that *accD*-C794 editing confers heat tolerance *in planta*. We also found that treatment with the product of acetyl coenzyme A carboxylase (ACCase) could allay mutant phenotypic characteristics and showed that a mutation in the *CAC3* gene for the α-subunit of ACCase was associated with dwarfism under heat stress. These observations indicate that defective *accD*-C794 editing may be intrinsic to reduced ACCase activity, thereby contributing to heat sensitivity. ACCase catalyzes the committed step of *de novo* fatty acid (FA) biosynthesis. FA content analysis revealed that unsaturated oleic (C18:1) and linoleic acids (C18:2) were low in the *accD*-C794 editing-defective mutant but high in the C794-edited *accD*-overexpressing plants compared with the wild type. Supplying exogenous C18:1 and C18:2 could rescue the mutant phenotype, suggesting that these FAs play an essential role in tolerance to heat stress. Transmission electron microscopy observations showed that heat stress seriously affected the membrane architecture in *accD* editing-defective mutants but not in *accD*-overexpressing plants. These results provide the first evidence that *accD*-C794 editing regulates FA biosynthesis for maintenance of membrane structural homeostasis under heat stress.

Key words: chloroplast RNA editing, *RARE1*, *accD*, fatty acid, heat stress

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INTRODUCTION

RNA editing involves post-transcriptional modifications, including nucleotide insertion, deletion, transitions, and transversions (Brennicke et al., 1999). In plants, one significant editing process within organellar mRNAs involves cytidine (C) to uridine (U) transition (C→U) (Chateigner-Boutin and Small, 2010; Yan et al., 2018; Small et al., 2020), with 619 and 43 editing sites identified in the mitochondrion and chloroplast of *Arabidopsis*, respectively (Ruwe et al., 2013; Leu et al., 2016). C→U editing events were originally proposed to involve an editosome complex that generally comprises pentatricopeptide repeat (PPR) proteins and non-PPR protein editing factors (Small

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et al., 2020; Sun et al., 2016; Yan et al., 2018). According to statistical analysis based on the plant editosome database (https://ngdc.cncb.ac.cn/ped/home), a total of 106 PPRs are involved in *Arabidopsis* organellar RNA editing, among which 47 PPRs participate in chloroplast RNA editing and the remainder are associated with mitochondria. In addition to PPRs, most RNA-editing sites require one or more non-PPR proteins, for example, multiple organellar RNA-editing factors (MORFs) (Takenaka et al., 2012; Bayer-Csaszar et al., 2017), organelle RNA recognition motif (ORRM) proteins (Shi et al., 2016), organelle zinc-finger (OZ) proteins (Sun et al., 2015), protoporphyrinogen IX oxidase 1 (PPO1) (Zhang et al., 2014), porphobilinogen deaminase HEMC (Huang et al., 2017), and chloroplast ribonucleoprotein 31A (CP31A) (Tillich et al., 2009). During RNA editing, transcriptome activation is considered to involve specific PPRs that interact at RNA sequences proximal to C targets aided by non-PPR recruitment of auxiliary cofactors either as editing activators or as regulators of editing efficiency (Small et al., 2020).

Recent evidence suggests that RNA editing is an essential response to abiotic stresses (Liu et al., 2018; Cui et al., 2019; Chu and Wei, 2020; Zhang et al., 2020). Interestingly, overall editing levels of several gene transcripts, such as *matK* and *ndhB*, decreased with increasing temperature in chloroplasts of *Vitis vinifera* (Zhang et al., 2020). Furthermore, transcriptome differences between heat-shocked and cold-stressed *Arabidopsis* showed that C→U editing rates were significantly affected by heat or cold stress (Chu and Wei, 2020), and editing of chloroplast *rps8*, *rpl2*, and *atpA* in *Oryza sativa* seems to correlate with cold-sensitive phenotypes (Cui et al., 2019; Liu et al., 2018). Nonetheless, the importance of RNA editing in plant growth is not apparent in genetic studies, and the molecular mechanisms that underlie the essential roles of RNA editing during temperature stress remain largely unknown.

In higher vascular plants, the ubiquitous unsaturated fatty acids (UFAs) C18:1, C18:2, and linolenic acid (C18:3) (Harwood, 1988; He et al., 2020) exert a biologically significant effect on organellar membrane structure, fluidity, and function under abiotic stress (He et al., 2018; Li et al., 2020). FA biosynthesis is initiated in chloroplasts and is tightly regulated by the *accD* gene (Thelen and Ohlrogge, 2002; Hölzl and Dörmann, 2019). This gene encodes the β-carboxyl transferase (β-CT) subunit of acetyl coenzyme A carboxylase (ACCase) (Sasaki et al., 1997), which is required for the conversion of acetyl-CoA into malonyl-CoA during the initial rate-limiting step of FA synthesis (Sasaki and Nagano, 2004). Two isoforms of ACCase are apparent within plant cells. The first is a cytosolic ACCase monomer, and the second is a heterotetrameric ACCase found in plastids (Sasaki et al., 1993; Konishi and Sasaki, 1994). Apart from the β-CT subunit, the heterotetrameric ACCase contains three additional subunits, including the biotin carboxyl carrier protein 1, biotin carboxylase, and the α-subunit of carboxyltransferase (α-CT), which are encoded by the nuclear genes *CAC1*, *CAC2*, and *CAC3*, respectively (Sasaki and Nagano, 2004; Yu et al., 2017). A previous study in *Nicotiana* sp. revealed that elevated *accD* expression was accompanied by increased ACCase content in plastids and significantly elevated levels of FAs in leaves (Madoka et al., 2002). Normal expression of *accD* requires RNA editing at the C794 and C1568 sites, which are located in the exon and the 3′ UTR, respectively, of the *accD* transcript (Chateigner-Boutin and Small, 2007). Editing at the former site is known to require the presence of a specific PPR protein, designated RARE1 (Robbins et al., 2009). However, the relationship between *accD*-C794 editing, ACCase, FA biosynthesis, and stress resistance remains largely unknown.

In the present study, we obtained an *Arabidopsis rare1* mutant with abolished *accD*-C794 editing and discovered that it exhibits a yellow and dwarf phenotype compared with the wild type under heat stress. Further investigation showed that heat tolerance in plants was strongly associated with the extent of *accD*-C794 editing. Moreover, exogenous malonyl-CoA application effectively rescued the *rare1* phenotype under heat stress, and mutation of the *CAC3* gene significantly contributed to heat-sensitive dwarfism in *Arabidopsis* plants. Thus, deficiency in *accD*-C794 editing dramatically influences plastid heteromeric ACCase activity. Further consequences of altered *accD*-C794 editing were observed in FA biosynthesis and the structural integrity of biological membranes, revealing that unsaturated C18:1 and C18:2 FAs play a vital role in membrane homeostasis under heat-stressed conditions. Our findings thus reveal an unexpected and novel role for C794 editing in the regulation of ACCase, FA synthesis, and heat-stress responses.

RESULTS

RARE1 loss of function causes a heat-sensitive growth defect

The PPR protein RARE1 is responsible for editing of the *accD*-C794 site (Robbins et al., 2009). To define the function of RARE1 in plants, we engineered two different mutants, each carrying a T-DNA insertion within *RARE1*, namely *rare1-1* (CS415940) and *rare1-2* (CS851454) (Figure 1A and Supplemental Figure 1). Subsequent DNA sequencing analysis showed that C794 editing of chloroplast *accD* transcripts was abolished in each case (Figure 1B). Both *rare1* lines displayed a phenotype not dissimilar to that of wild-type plants under normal growth conditions (Figure 1C), as noted elsewhere (Robbins et al., 2009). Strikingly, at an elevated temperature of 28°C, all *rare1* lines displayed yellow foliage and a slowed growth rate, whereas the more resilient wild-type plants retained typical green foliage. This mutant phenotype could be rescued by *pRARE1::RARE1-c-Myc* complementation (Figure 1C). Biochemical analysis revealed that there were no significant differences in chlorophyll content among the wild type, both *rare1* lines, and complementary transgenic plants at 22°C (Figure 1D). However, at an elevated temperature of 28°C, the contents of chlorophyll a, chlorophyll b, and total chlorophyll were significantly reduced in the *rare1 lines* compared with wild-type plants and the complemented lines (Figure 1E). Measurement of the Fv/Fm value further validated the above results *in vitro* (Figure 1F and 1G). Therefore, heat stress is severely detrimental to chlorophyll synthesis in *rare1* lines, which develop a yellow leaf phenotype.

RARE1 gene deficiency influences chloroplast gene expression

In our initial experiments, we assessed differences in expression among plastid-encoded RNA polymerase (PEP)-dependent genes

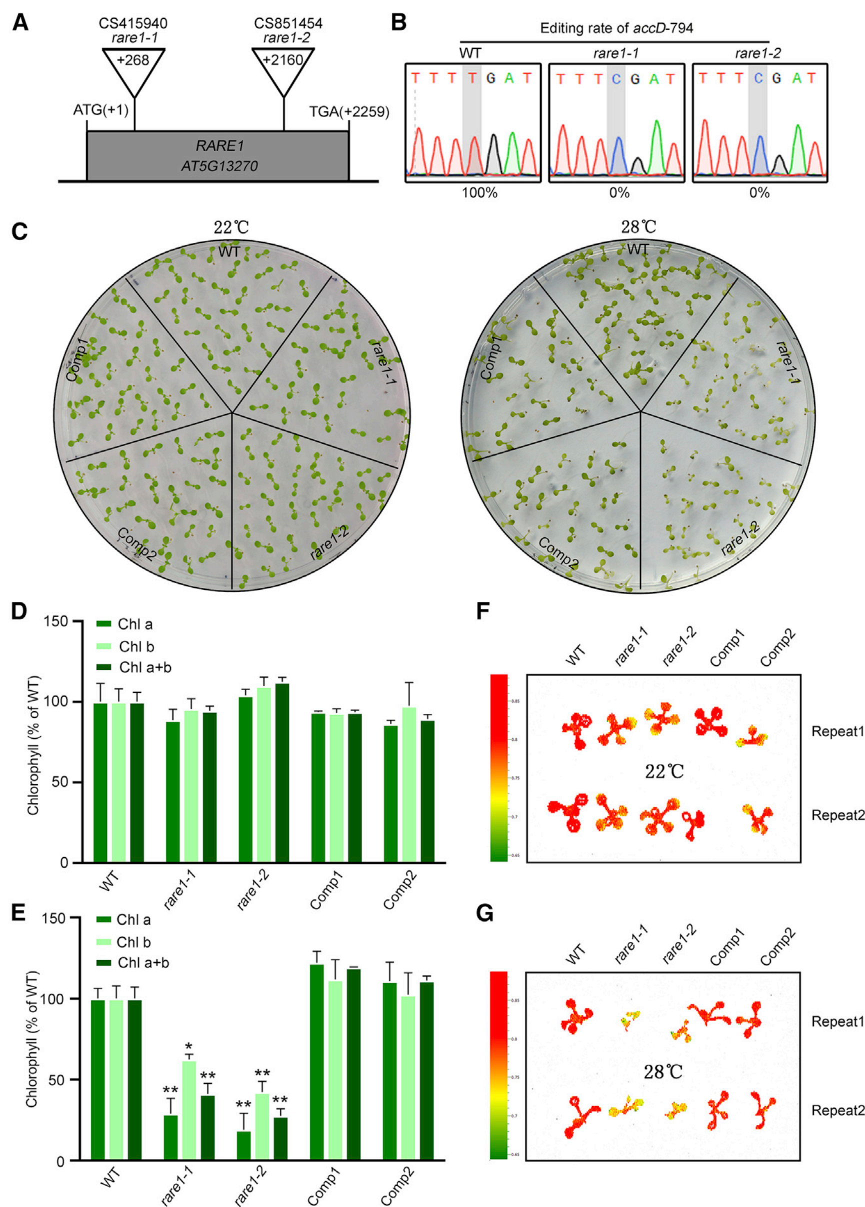


Figure 1. *Arabidopsis rare1* mutants with abolished *accD*-C794 editing displayed a heat-stress-sensitive phenotype.

(A) A schematic map of the *RARE1* locus, illustrating the T-DNA insertion sites within the *rare1* mutants.

(B) The editing rate of chloroplast *accD* transcripts at the C794 site in WT and *rare1* mutants.

(C) Seedling phenotypes of wild-type (WT), *rare1*, and pRARE1:RARE1-c-Myc transgenic plants (complemented) grown at 22°C or 28°C.

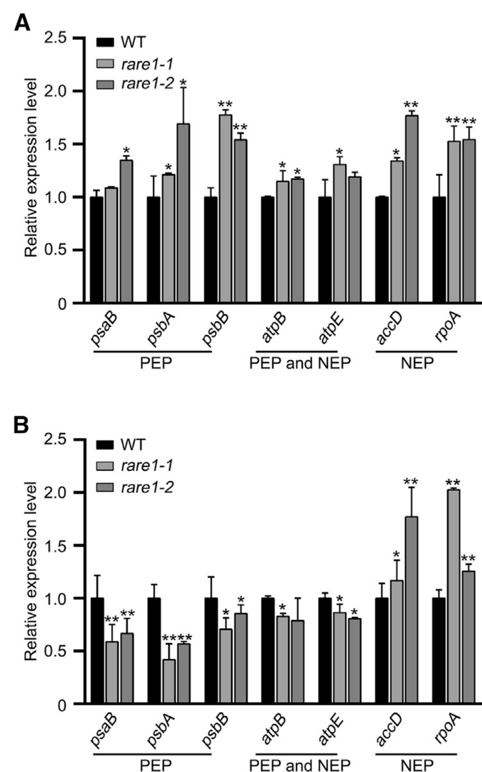
(D and E) Chlorophyll content in plants grown at 22°C **(D)** or 28°C **(E)**.

(F and G) Fv/Fm values of WT, *rare1*, and complemented lines grown at 22°C **(F)** or 28°C **(G)**. Error bars indicate SD from three independent repeats. Bars marked with an asterisk (Student's *t*-test; *p ≤ 0.05, **p ≤ 0.01) differ significantly from the WT sample.

(*psaB*, *psbA*, and *psbB*), nuclear-encoded RNA polymerase (NEP)-dependent genes (*accD* and *rpoA*), and genes that are both PEP- and NEP-dependent (*atpB* and *atpE*) in wild-type and *rare1* mutant *Arabidopsis* plants. Under normal growth conditions, almost all transcript levels of chloroplast genes were significantly higher in the *rare1* mutants than in the wild-type plants (Figure 2A). When expression levels of these genes were analyzed in each line cultivated at 28°C, PEP-related genes (*psaB*, *psbA*, *psbB*, *atpB*, and *atpE*) were repressed, whereas expression levels of *accD* and *rpoA* NEP-dependent genes were elevated (Figure 2B). Expression patterns of chloroplast transcripts in the *rare1* mutants grown at 28°C were similar to those in PEP-defective mutants described elsewhere (Gao et al., 2011; Yagi et al., 2012). In a series of immunoblotting experiments, we detected the effect of abnormal chloroplast gene transcription on accumulation of photosynthesis-related proteins in the *rare1* mutants, including the core subunit of photosystem I (PsaA), subunit H of NAD(P)H dehydrogenase (NdhH), subunit IV of the cytochrome b6f complex (PetD), and light-harvesting chlorophyll a/b complex II. In these experiments, PsaA, NdhH, and PetD levels were drastically increased in the *rare1* mutants compared with wild-type plants under normal growth conditions (Figure 2C). By contrast, the accumulation of these photosynthesis-related proteins was reduced in mutant plants under heat-stress conditions (Figure 2D). These results suggest that the *RARE1* mutation leads to significant defects in chloroplast development under heat stress and markedly increases the accumulation of chloroplast functional components under normal conditions.

Overexpression of the C794-edited *accD* gene enhances plant heat tolerance

To determine whether this *accD*-C794 editing deficiency confers heat-sensitive defects upon *rare1* mutants, we prepared a *35S:accD-His* construct in which the coding sequence of C794-edited *accD* was N-terminally fused with the chloroplast transit peptide from the protochlorophyllide oxidoreductase A (*PORA*) gene under the control of the CaMV 35S promoter (Figure 3A). The resultant transgenic plants overexpressing the C794-edited *accD* gene in the *rare1* mutant or wild-type background displayed a heat-tolerant phenotype compared with the original plants (Figure 3B and Supplemental Figure 2). Phenotype investigation of plants overexpressing C794-unedited *accD* under 28°C further demonstrated that the effect was not due to overexpression (Supplemental Figure 2). Furthermore, analysis of chlorophyll content showed that the transgenic plants with edited *accD* retained more chlorophyll than the *rare1* mutants under heat-stress conditions (Figure 3C). These results indicate that *accD*-C794 RNA editing is associated with heat tolerance in *Arabidopsis*. In addition, we found that three independent overexpressing lines of C794-edited *accD* (*accD* overexpressed



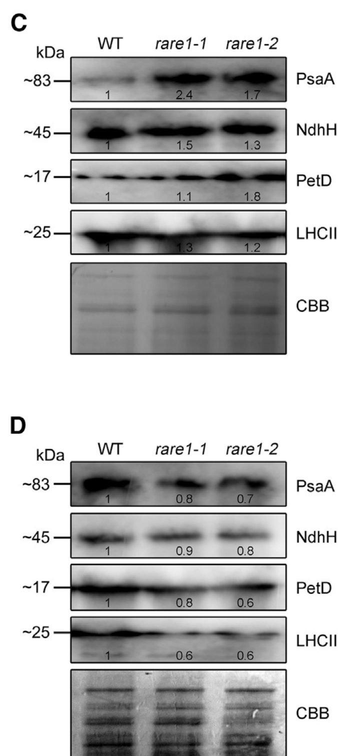


Figure 2. Effects of *RARE1* mutation on chloroplast gene expression.
(A) qRT–PCR analysis of chloroplast genes in WT and *rare1* plants cultured at 22°C. Representative genes, including plastid-encoded polymerase (PEP)-dependent, nuclear-encoded polymerase (NEP)-dependent, and PEP- and NEP-dependent genes encoded in chloroplasts, were used to analyze the impact of the *RARE1* mutation.
(B) Changes in transcript levels of chloroplast genes in WT and *rare1* plants grown at 28°C. Data represent the mean of three replicates ±SD.
(C and D) Detection of chloroplast protein accumulation in WT and *rare1* mutants grown at 22°C **(C)** or 28°C **(D)**. Bars denote standard errors.
Asterisks (*) in **(A)** and **(B)** indicate a significant difference from the WT (*$p \leq 0.05$; **$p \leq 0.01$).

[OE]) in the wild-type background exhibited significantly higher heat tolerance (Figure 3D and Supplemental Figure 3). Under heat stress, *accD*-OE plants grew more rapidly and better than wild-type plants (Figure 3D). We also compared heat-stress responses between wild-type and *accD*-OE plants at the reproductive stage and observed a greater abundance of siliques in the transgenic plants compared with the controls (Figure 3E). We also analyzed the RNA-editing rate at the *accD*-C794 site and the expression of *RARE1* after heat treatment. The *accD*-C794 editing rate and *RARE1* expression increased as the treatment time increased (Figure 3F and 3G). These findings suggest that the expression level of the edited *accD* gene contributes to heat tolerance in *Arabidopsis*.

The heat-sensitive phenotype of the *rare1* mutant is associated with a defect in ACCase activity

It is well documented that *accD* encodes a core subunit of ACCase required for FA biosynthesis (Sasaki et al., 1997). To examine the possibility that abolished RNA editing of the *accD* transcript results in ACCase inactivation under heat stress, we assessed the effects of malonyl-CoA supplements on the *rare1* phenotype. We observed that exogenous supplementation with malonyl-CoA alleviated the heat-induced defects of *rare1*, as indicated by a slight recovery from the yellow, dwarf phenotype on medium with a low concentration of malonyl-CoA lithium salt to near-wild-type characteristics at 28°C supplemented with 80 μg/ml malonyl-CoA lithium salt (Figure 4A). Levels of chlorophyll in *rare1* plants were highly correlated with the concentration of malonyl-CoA lithium salt under the described conditions (Figure 4B). An enzyme activity assay demonstrated that ACCase activity was lower in the *rare1* mutant compared with the wild type under heat-stress conditions (Figure 4C). To further investigate the association between the heat sensitivity of *rare1* and ACCase dysfunction, we analyzed the growth phenotype of wild-type seedlings treated with an inhibitor of plastid ACCase. Inhibition of ACCase caused a dwarf phenotype at 28°C but not at 22°C (Figure 4D). We also identified and characterized a T-DNA insertion line of the *CAC3* gene encoding the α-CT subunit of ACCase, which we named *cac3-1*. Sequencing analysis revealed that the T-DNA insertion in the non-coding 3′ UTR of *CAC3* significantly reduced expression from neighboring sequences but not the coding region (Supplemental Figure 4). The *cac3-1* mutants displayed near-wild-type phenotypes at 22°C but adopted a dwarf habit at 28°C (Figure 4E). A novel allelic mutant of *CAC3* (*cac3-2*) obtained by CRISPR-Cas9 genome editing also exhibited a severe dwarf phenotype under heat-stress conditions (Figure 4F and Supplemental Figure 5). These findings suggest that loss of *accD*-C794 editing directly impairs the activity of ACCase, which directly manifests as the heat-sensitive phenotype of *rare1* mutants.

Levels of the UFAs C18:1 and C18:2 correlate with heat tolerance

UFAs play an essential role in plant adaptation to environmental stresses and presumably contribute to the heat sensitivity of *rare1* plants. We therefore made gas chromatography-mass spectrometry comparisons of UFA levels between wild-type and *rare1* plants, and the abundances of C18:1, C18:2, and C18:3 UFAs differed significantly between the genotypes. Levels of C18:1 and C18:2 were lower in the *rare1* mutants, whereas the C18:3 content was higher (*cf*. wild type; Figure 5A). Furthermore, when exogenous C18:1 and C18:2 were applied to *rare1* plants at a concentration of 1 μg/ml under heat stress, the development of the *rare1* yellow phenotype was less pronounced (Figure 5B and Supplemental Figure 6B). By contrast, no phenotype differences were observed between the wild type and *rare1* under normal conditions (Supplemental Figure 6A). We also measured chlorophyll content in *rare1* mutants cultured at 28°C in the presence of

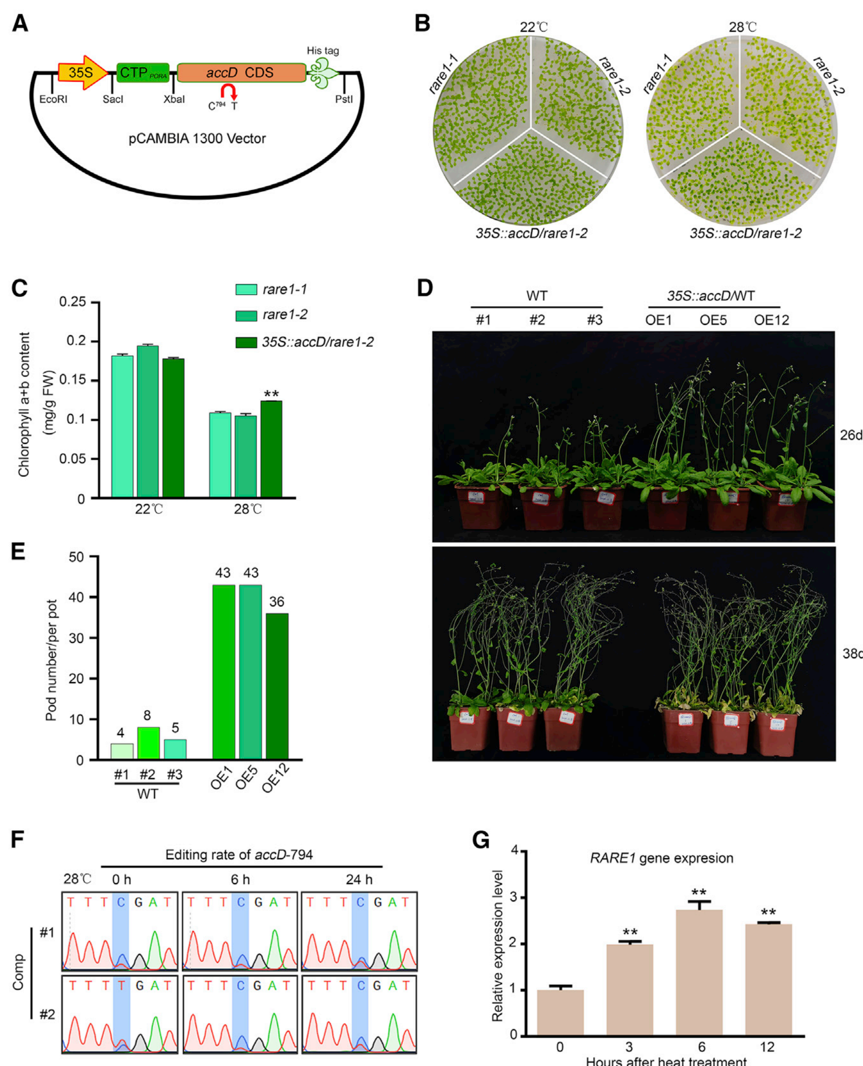


Figure 3. Overexpression of the C794-edited *accD* gene conferred heat tolerance.
(A) Schematic illustration of the *accD*-fusion construct. Expression of the edited *accD* gene fused with the chloroplast transit peptide of *RPOA* and a His-tag is driven by the 35S promoter.
(B) Phenotype of C794-edited *accD*-overexpressing transgenic plants in the *rare1* background.
(C) Chlorophyll contents in transgenic seedlings grown at 22°C or 28°C. Data represent the mean of three replicates ±SD. Asterisks (*) indicate a statistical difference (**$p \leq 0.01$).
(D) Heat-tolerance assay of three edited *accD* transgenic lines in the WT background.
(E) The number of fertile siliques in the WT and transgenic plants under heat stress.
(F) Effect of heat treatment on editing rate at the *accD*-C794 site in two independent RARE1-complemented plants with incomplete *accD* editing.
(G) Effect of heat treatment on *RARE1* gene expression.

C18:1 and C18:2 supplements and found that levels of chlorophyll in the *rare1* mutants increased compared with the control plants (Figure 5C). Transgenic plants overexpressing the C794-edited *accD* gene in the wild-type background exhibited enhanced heat tolerance (Figure 3D). Therefore, we also measured FA levels in the seeds of three independent transgenic lines and found that their total FA content was 11.7%–28.3% higher than that of wild-type seeds (Supplemental Figure 7). Further analysis of the FA composition revealed that C18:1 and C18:2 levels were significantly higher in seeds of the transgenic lines (Figure 5D). These results suggest that C18:1 and C18:2 UFAs are responsible for RNA-editing-related heat tolerance in *Arabidopsis*.

Heat stress causes membrane damage in *rare1* mutant plants

UFAs are important components of phospholipids in biomembranes and help to maintain membrane fluidity. We examined the structures of organelles and plasma membranes in wild-type, *rare1*, and C794-edited *accD*-OE leaf cells using transmission electron microscopy (TEM) (Figure 6). There were no significant differences in chloroplast and plasma membrane structure among wild-type, *rare1,* and C794-edited *accD*-OE plants under normal conditions (Figure 6A and 6B). Interestingly, chloroplasts in wild-type and OE cells appeared as predominantly lunate entities with abundant thylakoid membranes. However, corresponding plastids in *rare1* mutants appeared as partially lysed, morphologically irregular organelles with a lower abundance of thylakoid membranes (Figure 6C). We also examined plasmalemma structures in cells from the same plants at 28°C and noted that whereas cellular surfaces in wild-type and *accD*-OE plants stained deep black, staining of the corresponding *rare1* membranes was significantly less pronounced (Figure 6D). These results suggest that biological membrane integrity is maintained under heat stress in wild-type and OE plants, but not in *rare1* plants.

DISCUSSION

The chloroplast *accD* gene encodes the β-CT subunit of heteromeric ACCase, which catalyzes the first committed step in FA biosynthesis. After transcription, *accD* transcripts undergo RNA editing at two sites, C794 and C1568. In fact, almost all *accD* transcripts in *Arabidopsis* exist in the C794-edited form. C794-edited *accD* seems to be more beneficial to plant growth. Nevertheless, Robbins et al. (2009) reported that an *Arabidopsis* mutant lacking *accD*-C794 editing exhibits a wild-type phenotype under growth-room conditions. To date, the function and importance of *accD*-C794 editing in plants remain unclear. We have engineered two alleles of *RARE1* that are mutated in *accD*-C794 editing capabilities, and the corresponding plants display a yellow, dwarf phenotype when cultivated at 28°C, suggesting

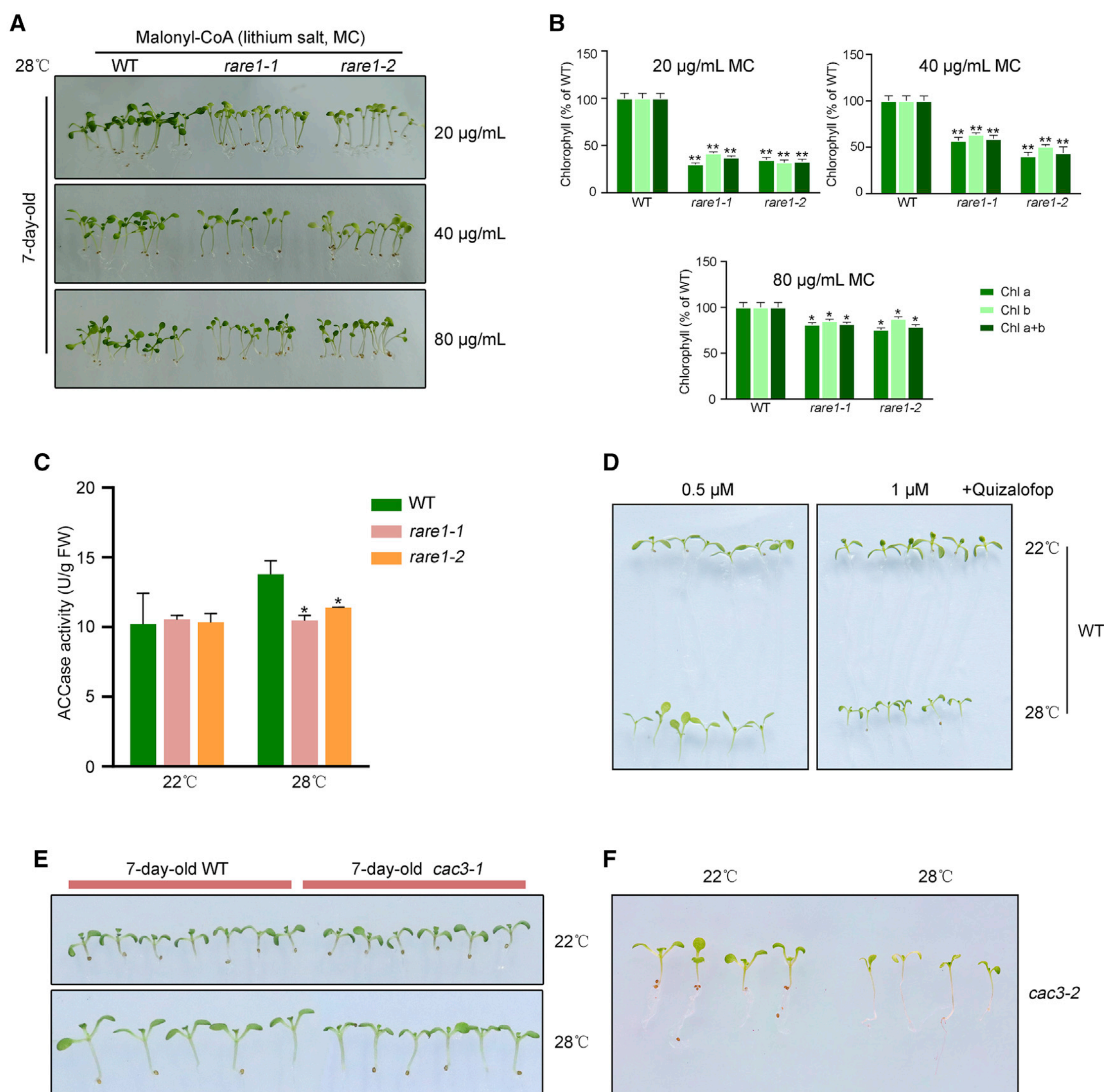


Figure 4. ACCase activity is tightly correlated with heat tolerance.

(A) Phenotypic analysis of *rare1* mutants grown on Murashige and Skoog medium supplemented with varying concentrations of malonyl-CoA lithium salt.

(B) Chlorophyll content of *rare1* plants grown with malonyl-CoA supplementation. Asterisks (*) indicate a significant difference between the WT and the *rare1* mutants (*$p \leq 0.05$; **$p \leq 0.01$).

(C) ACCase activity in the WT and the *rare1* mutants at 22°C or 28°C.

(D) Effect of a plastid ACCase inhibitor on the phenotype of the WT under 22°C or 28°C.

(E and F) Growth phenotypes of *cac3* mutants at 22°C and 28°C.

that editing is necessary for normal responses to heat stress. At the same time, it is possible that other RARE1-mediated processes may be involved in the heat-induced phenotype of the *rare1* mutants. To address this issue, we overexpressed C794-edited *accD* in both *rare1* mutants and the wild type and observed enhanced heat tolerance compared with the control plants (Figure 3). This evidence provides support for the notion that *accD*-C794 editing confers tolerance to heat stress in *Arabidopsis*.

Editing at the C794 site underlies the S265→L substitution in the AccD protein, but it is still considered to be non-essential for full heteromeric ACCase enzyme activity because the abolition of C794 RNA editing does not affect plant growth, ACCase activity, and total seed FA content under normal conditions (Figures 1C and 4C and Supplemental Figure 8). It is possible that levels of cytoplasmic homomeric ACCase are sufficient to satisfy the FA demand for plant growth under normal conditions. Here, we also demonstrated that *Arabidopsis* plants carrying a mutation in the α-CT subunit of ACCase encoded by the *CAC3* gene display a phenotype similar to that of wild-type plants under normal conditions (Figure 4E). Despite this result, previous studies have shown that mutations in the *ACC1* and *ACC2* genes, which encode two isoforms of homomeric ACCase, produce a lethal embryonic phenotype (Baud et al., 2003). Thus, we speculate that homomeric ACCase has greater biological significance than its heterotetrameric counterpart for plant growth under normal conditions.

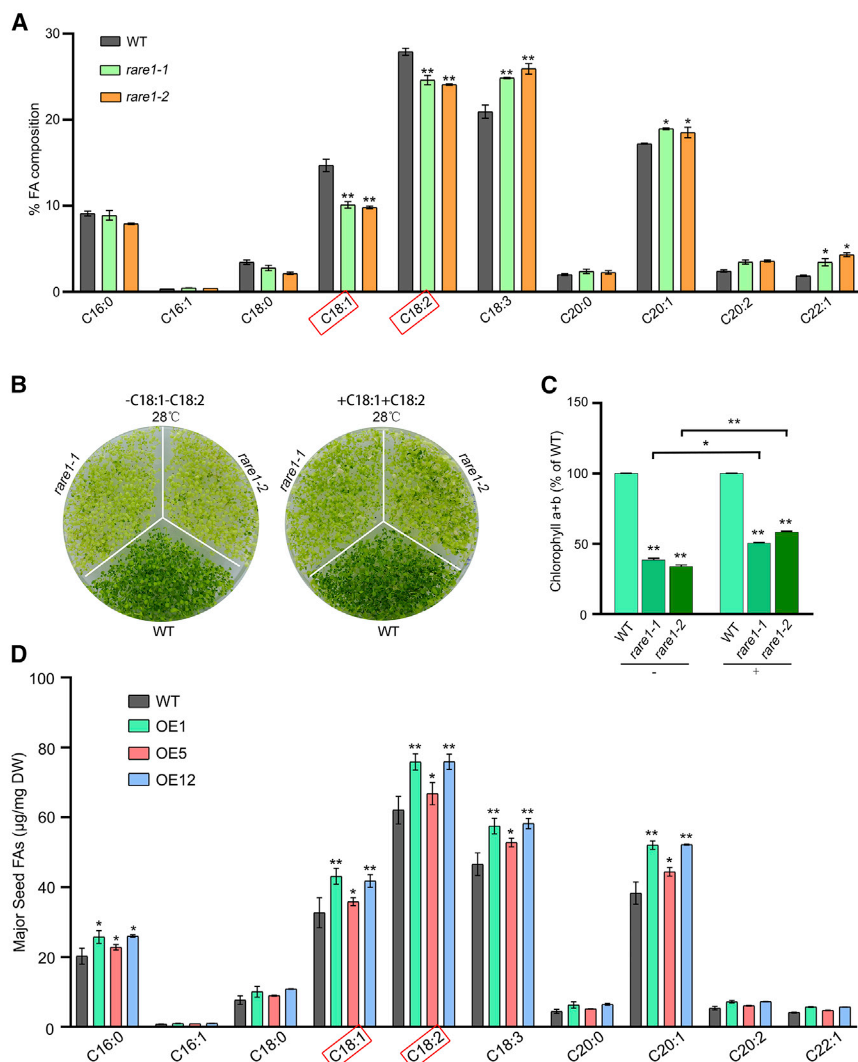


Figure 5. Analysis of fatty acid composition in *rare1* mutants and *accD*-overexpressing lines.

(A) Comparison of fatty acid composition between the WT and the *rare1* mutants.

(B) Phenotypic observation of the WT and the *rare1* mutants grown with C18:1 and C18:2 supplementation at 28°C.

(C) Changes in chlorophyll content of the WT and the *rare1* mutants grown with or without C18:1 and C18:2. The chlorophyll content of WT seedlings is set to 100%.

(D) Comparison of fatty acid composition between the WT and the *accD*-overexpressing (OE) plants in the WT background. Asterisks (*) indicate significantly higher or lower levels relative to the WT (*$p \leq 0.05$; **$p \leq 0.01$).

The plastid heteromeric ACCase has primary sequence similarity to the prokaryotic *Escherichia coli* isomer and is considered to be of cyanobacterial evolutionary origin (Nikolau et al., 2003). Previous reports have shown that a mutation in the BCCP protein subunit of ACCase in *E. coli* confers a temperature-sensitive phenotype (Cronan, 2001). Coupled with observations that *Arabidopsis cac3* mutants lacking the ACCase α-CT subunit are also phenotypically heat sensitive (Figure 4E and 4F), these reports lead us to surmise that the plastid heterotetrameric ACCase plays a central role in normal plant growth under heat stress. This notion is further supported by the finding that the *rare1* mutants showed significantly lower lipid contents under heat stress (Supplemental Figure 9). It is noteworthy that *Arabidopsis* plants carrying the *CAC3* mutation and *accD*-C794 editing deficiency also showed phenotypic similarities during heat stress and that exogenous supplementation with malonyl-CoA, the product of ACCase, partially rescued the phenotype of C794-editing-deficient plants (Figure 4A). These results suggest that *accD*-C794 editing deficiency reduces the plastid ACCase activity. In addition, the editing rate of *accD*-C794 appears to be directly proportional to the heat tolerance of plants (Figure 3F). These observations suggest that heteromeric ACCase confers heat tolerance and that *accD*-C794 editing is critical for the function of heteromeric ACCase under heat stress.

C18:1, C18:2, and C18:3 are the predominant UFAs in most plants (Harwood, 1988) and are associated with both abiotic and biotic plant stresses (He et al., 2020). We observed a decrease in C18:1 and C18:2 UFA content in the *accD*-C794-editing-deficient plants, whereas levels of these UFAs increased in the C794-edited *accD*-OE plants (Figure 5A and 5D). In addition, the *accD*-C794-editing-deficient mutants are phenotypically heat sensitive, whereas edited *accD*-OE plants exhibit enhanced heat tolerance (Figures 1C and 3). These findings suggest that the heat-stress phenotype is related to altered C18:1 and C18:2 contents in these plants. This notion is further supported by C18:1 and C18:2 supplementation studies of the *accD*-C794-editing-deficient mutants under heat stress (Figure 5B and Supplemental Figure 6B).

Membrane lipids contain high levels of UFAs, which are essential for homeostatic maintenance of membrane structure. Previous reports have indicated that the plasmalemmas surrounding thermotolerant yeast cells are enriched in C18:1 content, with proportionately lower levels of C18:2 and C18:3, in that order (Swan and Watson, 1999). Moreover, C18:1 is reported to be crucial for the maintenance of cell membrane integrity and fluidity, thus contributing to improved survival rates of *Lactobacillus plantarum* after freeze drying (Wang et al., 2020). In this study, *accD*-C794-editing-deficient mutants with low proportions of C18:1 and C18:2 showed a heat-induced yellow, dwarf phenotype. In addition, TEM observations of heat-stress-treated plant tissues revealed morphologically compromised chloroplast membrane integrity and partial lipid staining of cellular plasma membranes in these plants (Figure 6), highlighting the importance of C18:1 and C18:2 for biological membrane system integrity under heat stress.

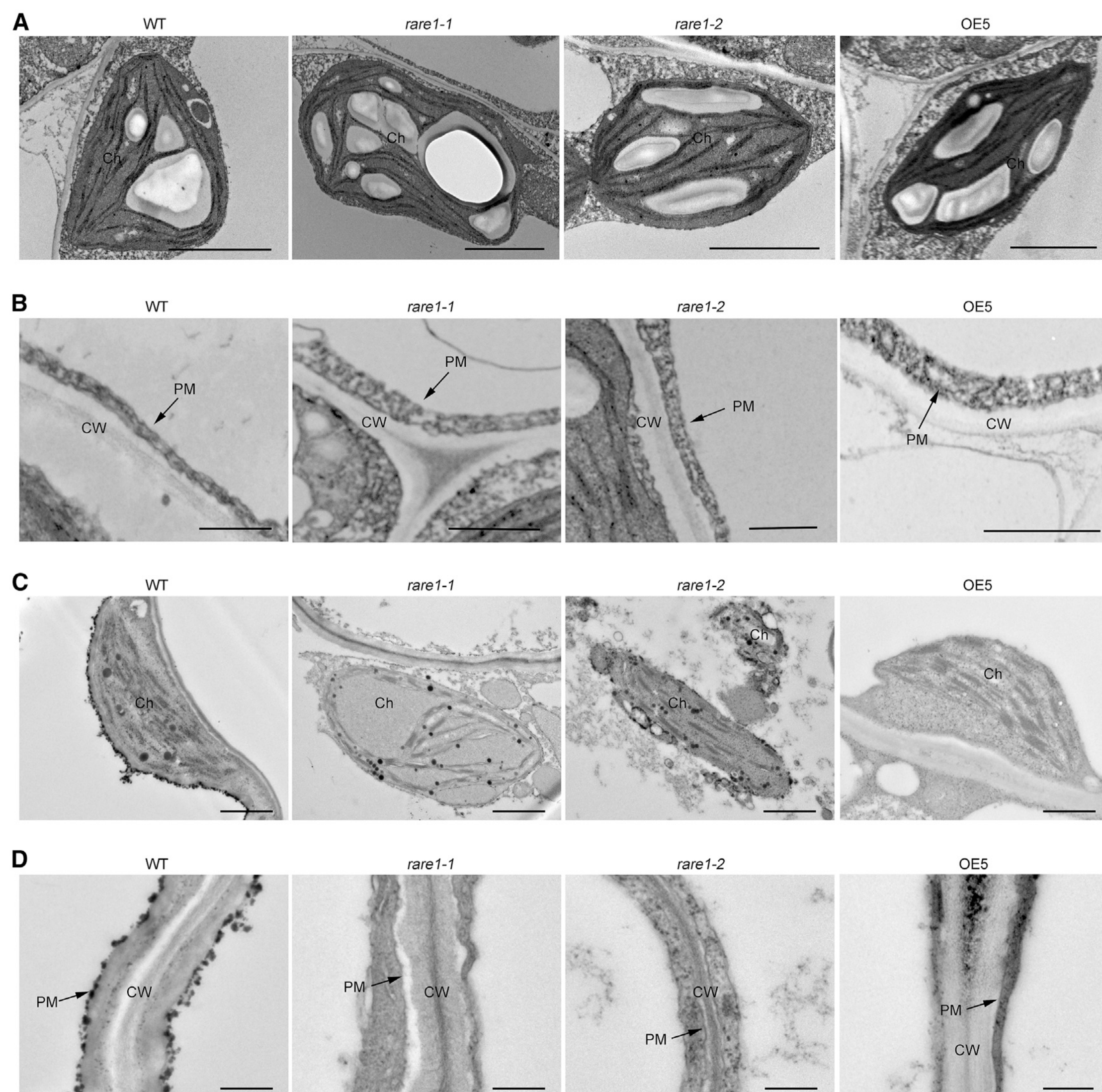


Figure 6. Transmission electron micrographs of biological membranes in the WT, the *rare1* mutants, and the edited *accD*-OE transgenic plants.

(A and C) Chloroplast ultrastructure of the WT, the *rare1* mutants, and the OE plants grown at 22°C **(A)** or 28°C **(C)**. Ch, chloroplast. OE, the C794-edited *accD*-OE transgenic line.

(B and D) Plasma membrane structure of the WT, the *rare1* mutants, and the *accD*-OE plants under 22°C **(B)** or 28°C **(D)**. PM, plasma membrane; CW, cell wall.

Scale bars: 2 μm in **(A)** and **(C)** and 500 nm in **(B)** and **(D)**.

In summary, the data presented here suggest that *accD*-C794 editing positively affects ACCase activity; this activity is essential for FA biosynthesis, which in turn correlates directly with the proportions of C18:1 and C18:2 UFAs that are integral to the biological membrane homeostasis required for correct physiological functioning and heat tolerance (Figure 7).

METHODS

Plant materials and growth conditions

The *rare1* (CS415940 and CS851454) mutants and *cac3* mutant (SALK_207356) used in this study were obtained from the Arabidopsis Biological Resource Center. Individual mutant plants were genotyped by PCR from genomic DNA with oligonucleotide primers designed in locations flanking both ends of the T-DNA insertion, along with a third primer located on the left border of the T-DNA insertion. For phenotypic characterization, chlorophyll detection, and immunoblot analyses, *Arabidopsis* seeds were surface sterilized, plated on Murashige and Skoog medium (Sigma) containing 1% sucrose, incubated at 4°C for 2 days, and then grown under long-day conditions (16 h light and 8 h darkness) at 22°C or 28°C for 7 days. For exogenous application experiments, seedlings were grown on 1% sucrose-containing Murashige and Skoog medium with malonyl-CoA lithium salt (Sigma) or UFAs (Aladdin) under the above-mentioned growth conditions.

Chloroplast RNA-editing analysis

Total RNA was purified using the TRIzol reagent (Thermo Fisher Scientific), and first-strand cDNA was synthesized using a HiScript III 1st Strand cDNA Synthesis Kit (+gDNA wiper) (Vazyme). Specific cDNA fragments containing editing sites were amplified and sequenced directly.

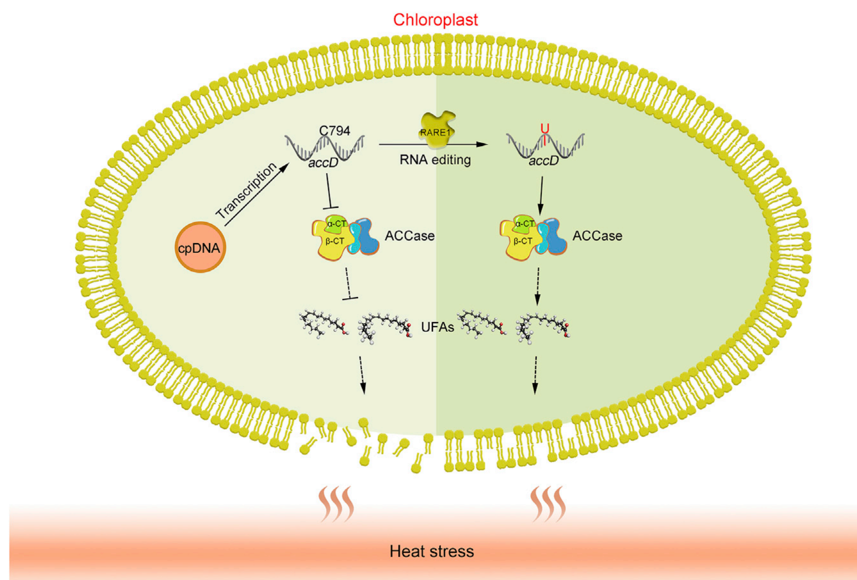


Figure 7. A working model showing how *accD*-C794 editing influences the regulation of ACCase activity and fatty acid biosynthesis.
Chloroplast *accD* transcripts undergo C→U RNA editing at the C794 site, which depends on the PPR protein RARE1. Loss of RARE1 function causes defects in C794 editing of *accD* transcripts. The extent of editing of *accD*, which encodes the α-CT of heterotetrametric plastid ACCase, is positively correlated with ACCase activity, thereby influencing UFA biosynthesis and ultimately regulating plastid membrane integrity under heat stress.

RNA-editing efficiency was estimated from the relative height of nucleotide peaks. The primer sequences are listed in Supplemental Table 1.

Quantitative RT-PCR

Quantitative RT-PCR was carried out as described previously (Huang et al., 2020). The *AtActin2* gene was used as an internal control. The sequence information used for quantitative RT-PCR is presented in Supplemental Table 1.

Immunoblot analyses

Total protein was extracted from the wild type, *rare1* mutants, and *accD*-OE transgenic plants for immunological detection as described previously (Huang et al., 2020). The amount of total protein was measured by a Bradford assay, and 40 μg total protein was loaded for each sample. The polyclonal antibodies for PsaA, NdhH, PetD, and light-harvesting chlorophyll a/b complex II were obtained as a gift from Dr. Hai-Bo Xiong (The Institute of Botany, Chinese Academy of Sciences). The anti-His antibody was obtained from Abcam.

Lipid analysis

Total FAs were extracted and converted to FA methyl esters in methanol solution containing 5% sulfuric acid. Samples were treated with ultrasound for 30 min at 42°C and then incubated at 65°C for 3 h. After that, FA methyl esters were extracted into hexane and 0.9% (w/v) NaCl and analyzed on an Agilent Technologies GCMS-TQ8050 system with a 100 m × 0.25 mm ID SH-Rt-2560 column (Shimadzu).

TEM

Leaves from 7-day-old wild-type, *rare1*, and *accD*-OE plants were fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.4) followed by osmium tetroxide and then dehydrated in an ethanol series. Ultrathin sections were obtained using a diamond knife and mounted on copper grids. The grids were then stained with uranyl acetate and alkaline lead citrate and examined using a JEM-1400 (JEOL) transmission electron microscope.

ACCase activity analysis

Fresh tissue (about 0.1 g) of whole seedlings from 7-day-old wild-type and *rare1* mutants was ground thoroughly using a TissueLyser II (Qiagen). The ACCase activity assay was carried out using an ACC assay kit (Zcibio) according to the manufacturer's descriptions. Absorbance at 660 nm was measured with a Multimode Microplate Reader (Tecan).

SUPPLEMENTAL INFORMATION

Supplemental information is available at *Plant Communications Online*.

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AUTHOR CONTRIBUTIONS

C.H. and L.-T.X. conceived the project and designed the research. C.H., D.L., Z.-A.L., Z.-F.L., and H.-O.L. performed experiments. C.H., Y.S., Q.L., and R.-Z.W. analyzed data. C.H., D.P.M., and L.-T.X. wrote the paper, which all authors edited and approved.

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REFERENCES

Baud, S., Guyon, V., Kronenberger, J., Wuillème, S., Miquel, M., Caboche, M., Lepiniec, L., and Rochat, C. (2003). Multifunctional acetyl-CoA carboxylase 1 is essential for very long chain fatty acid elongation and embryo development in Arabidopsis. Plant J. **33**:75–86. https://doi.org/10.1046/j.1365-313x.2003.016010.x.

Bayer-Császár, E., Haag, S., Jörg, A., Glass, F., Härtel, B., Obata, T., Meyer, E.H., Brennicke, A., and Takenaka, M. (2017). The conserved domain in MORF proteins has distinct affinities to the PPR and E elements in PPR RNA editing factors. Biochim. Biophys. Acta. Gene Regul. Mech. **1860**:813–828. https://doi.org/10.1016/j.bbagrm.2017.05.004.

Brennicke, A., Marchfelder, A., and Binder, S. (1999). RNA editing. FEMS Microbiol. Rev. **23**:297–316.

Chateigner-Boutin, A.L., and Small, I. (2007). A rapid high-throughput method for the detection and quantification of RNA editing based on high-resolution melting of amplicons. Nucleic Acids Res. **35**:e114. https://doi.org/10.1093/nar/gkm640.

Chateigner-Boutin, A.L., and Small, I. (2010). Plant RNA editing. RNA Biol. **7**:213–219. https://doi.org/10.4161/rna.7.2.11343.

Chu, D., and Wei, L. (2020). Reduced C-to-U RNA editing rates might play a regulatory role in stress response of Arabidopsis. J. Plant Physiol. **244**:153081. https://doi.org/10.1016/j.jplph.2019.153081.

Cronan, J.E., Jr. (2001). The biotinyl domain of Escherichia coli acetyl-CoA carboxylase. Evidence that the "thumb" structure id essential and that the domain functions as a dimer. J. Biol. Chem. **276**:37355–37364. https://doi.org/10.1074/jbc.M106353200.

Cui, X., Wang, Y., Wu, J., Han, X., Gu, X., Lu, T., and Zhang, Z. (2019). The RNA editing factor DUA1 is crucial to chloroplast development at low temperature in rice. New Phytol. **221**:834–849. https://doi.org/10.1111/nph.15448.

Gao, Z.P., Yu, Q.B., Zhao, T.T., Ma, Q., Chen, G.X., and Yang, Z.N. (2011). A functional component of the transcriptionally active chromosome complex, Arabidopsis pTAC14, interacts with pTAC12/HEMERA and regulates plastid gene expression. Plant Physiol. **157**:1733–1745. https://doi.org/10.1104/pp.111.184762.

Hölzl, G., and Dörmann, P. (2019). Chloroplast lipids and their biosynthesis. Annu. Rev. Plant Biol. **70**:51–81. https://doi.org/10.1146/annurev-arplant-050718-100202.

Harwood, J.L. (1988). Fatty acid metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. **39**:101–138. https://doi.org/10.1146/annurev.pp.39.060188.000533.

He, M., He, C.Q., and Ding, N.Z. (2018). Abiotic stresses: general defenses of land plants and chances for engineering multistress tolerance. Front. Plant Sci. **9**:1771. https://doi.org/10.3389/fpls.2018.01771.

He, M., Qin, C.X., Wang, X., and Ding, N.Z. (2020). Plant unsaturated fatty acids: biosynthesis and regulation. Front. Plant Sci. **11**:390. https://doi.org/10.3389/fpls.2020.00390.

Huang, C., David, M.P., Cao, Z.-L., and Xiao, L.-T. (2020). Mutation of chloroplast CHLM contributes to down-regulation of multiple stress response genes in Arabidopsis. Plant Growth Regul. **91**:209–219. https://doi.org/10.1007/s10725-020-00600-9.

Huang, C., Yu, Q.-B., Li, Z.-R., Ye, L.-S., Xu, L., and Yang, Z.-N. (2017). Porphobilinogen deaminase HEMC interacts with the PPR-protein AtECB2 for chloroplast RNA editing. Plant J. **92**:546–556. https://doi.org/10.1111/tpj.13672.

Konishi, T., and Sasaki, Y. (1994). Compartmentalization of two forms of acetyl-CoA carboxylase in plants and the origin of their tolerance toward herbicides. Proc. Natl. Acad. Sci. USA **91**:3598–3601. https://doi.org/10.1073/pnas.91.9.3598.

Leu, K.-C., Hsieh, M.-H., Wang, H.-J., Hsieh, H.-L., and Jauh, G.-Y. (2016). Distinct role of Arabidopsis mitochondrial P-type pentatricopeptide repeat protein-modulating editing protein, PPME, in nad1 RNA editing. RNA Biol. **13**:593–604. https://doi.org/10.1080/15476286.2016.1184384.

Li, J., Liu, L.N., Meng, Q., Fan, H., and Sui, N. (2020). The roles of chloroplast membrane lipids in abiotic stress responses. Plant Signal. Behav. **15**:1807152. https://doi.org/10.1080/15592324.2020.1807152.

Liu, X., Lan, J., Huang, Y., Cao, P., Zhou, C., Ren, Y., He, N., Liu, S., Tian, Y., Nguyen, T., et al. (2018). WSL5, a pentatricopeptide repeat protein, is essential for chloroplast biogenesis in rice under cold stress. J. Exp. Bot. **69**:3949–3961. https://doi.org/10.1093/jxb/ery214.

Madoka, Y., Tomizawa, K.I., Mizoi, J., Nishida, I., Nagano, Y., and Sasaki, Y. (2002). Chloroplast transformation with modified accD operon increases acetyl-CoA carboxylase and causes extension of leaf longevity and increase in seed yield in tobacco. Plant Cell Physiol. **43**:1518–1525. https://doi.org/10.1093/pcp/pcf172.

Nikolau, B.J., Ohlrogge, J.B., and Wurtele, E.S. (2003). Plant biotin-containing carboxylases. Arch. Biochem. Biophys. **414**:211–222. https://doi.org/10.1016/s0003-9861(03)00156-5.

Robbins, J.C., Heller, W.P., and Hanson, M.R. (2009). A comparative genomics approach identifies a PPR-DYW protein that is essential for C-to-U editing of the Arabidopsis chloroplast accD transcript. RNA **15**:1142–1153. https://doi.org/10.1261/rna.1533909.

Ruwe, H., Castandet, B., Schmitz-Linneweber, C., and Stern, D.B. (2013). Arabidopsis chloroplast quantitative editotype. FEBS Lett. **587**:1429–1433. https://doi.org/10.1016/j.febslet.2013.03.022.

Sasaki, Y., and Nagano, Y. (2004). Plant acetyl-CoA carboxylase: structure, biosynthesis, regulation, and gene manipulation for plant breeding. Biosci. Biotechnol. Biochem. **68**:1175–1184. https://doi.org/10.1271/bbb.68.1175.

Sasaki, Y., Kozaki, A., and Hatano, M. (1997). Link between light and fatty acid synthesis: thioredoxin-linked reductive activation of plastidic acetyl-CoA carboxylase. Proc. Natl. Acad. Sci. USA **94**:11096–11101.

Sasaki, Y., Hakamada, K., Suama, Y., Nagano, Y., Furusawa, I., and Matsuno, R. (1993). Chloroplast-encoded protein as a subunit of acetyl-CoA carboxylase in pea plant. J. Biol. Chem. **268**:25118–25123. https://doi.org/10.1016/s0021-9258(19)74577-3.

Shi, X., Bentolila, S., and Hanson, M.R. (2016). Organelle RNA recognition motif-containing (ORRM) proteins are plastid and mitochondrial editing factors in Arabidopsis. Plant Signal. Behav. **11**:e1167299. https://doi.org/10.1080/15592324.2016.1167299.

Small, I.D., Schallenberg-Rüdinger, M., Takenaka, M., Mireau, H., and Ostersetzer-Biran, O. (2020). Plant organellar RNA editing: what 30 years of research has revealed. Plant J. **101**:1040–1056. https://doi.org/10.1111/tpj.14578.

Sun, T., Bentolila, S., and Hanson, M.R. (2016). The unexpected diversity of plant organelle RNA editosomes. Trends Plant Sci. **21**:962–973. https://doi.org/10.1016/j.tplants.2016.07.005.

Sun, T., Shi, X., Friso, G., Van Wijk, K., Bentolila, S., and Hanson, M.R. (2015). A zinc finger motif-containing protein is essential for chloroplast RNA editing. PLoS Genet. **11**:e1005028. https://doi.org/10.1371/journal.pgen.1005028.

Swan, T.M., and Watson, K. (1999). Stress tolerance in a yeast lipid mutant: membrane lipids influence tolerance to heat and ethanol independently of heat shock proteins and trehalose. Can. J. Microbiol. **45**:472–479. https://doi.org/10.1139/w99-033.

Takenaka, M., Zehrmann, A., Verbitskiy, D., Kugelmann, M., Härtel, B., and Brennicke, A. (2012). Multiple organellar RNA editing factor (MORF) family proteins are required for RNA editing in mitochondria and plastids of plants. Proc. Natl. Acad. Sci. USA **109**:5104–5109. https://doi.org/10.1073/pnas.1202452109.

Thelen, J.J., and Ohlrogge, J.B. (2002). Metabolic engineering of fatty acid biosynthesis in plants. Metab. Eng. **4**:12–21. https://doi.org/10.1006/mben.2001.0204.

Tillich, M., Hardel, S.L., Kupsch, C., Armbruster, U., Delannoy, E., Gualberto, J.M., Lehwark, P., Leister, D., Small, I.D., and Schmitz-Linneweber, C. (2009). Chloroplast ribonucleoprotein CP31A is required for editing and stability of specific chloroplast mRNAs. Proc. Natl. Acad. Sci. USA **106**:6002–6007. https://doi.org/10.1073/pnas.0808529106.

Wang, G., Chen, P., Yu, X., Xia, Y., Yan, L.T., and Ai, L. (2020). C18:1 improves the freeze-drying resistance of Lactobacillus plantarum by maintaining the cell membrane. ACS Appl. Bio Mater. **3**:4933–4940. https://doi.org/10.1021/acsabm.0c00444.

Yagi, Y., Ishizaki, Y., Nakahira, Y., Tozawa, Y., and Shiina, T. (2012). Eukaryotic-type plastid nucleoid protein pTAC3 is essential for

transcription by the bacterial-type plastid RNA polymerase. Proc. Natl. Acad. Sci. USA **109**:7541–7546. https://doi.org/10.1073/pnas.1119403109.

Yan, J., Zhang, Q., and Yin, P. (2018). RNA editing machinery in plant organelles. Sci. China Life Sci. **61**:162–169. https://doi.org/10.1007/s11427-017-9170-3.

Yu, Q., Lutz, K.A., and Maliga, P. (2017). Efficient plastid transformation in Arabidopsis. Plant Physiol. **175**:186–193. https://doi.org/10.1104/pp.17.00857.

Zhang, A., Jiang, X., Zhang, F., Wang, T., and Zhang, X. (2020). Dynamic response of RNA editing to temperature in grape by RNA deep sequencing. Funct. Integr. Genomics **20**:421–432. https://doi.org/10.1007/s10142-019-00727-7.

Zhang, F., Tang, W., Hedtke, B., Zhong, L., Liu, L., Peng, L., Lu, C., Grimm, B., and Lin, R. (2014). Tetrapyrrole biosynthetic enzyme protoporphyrinogen IX oxidase 1 is required for plastid RNA editing. Proc. Natl. Acad. Sci. USA **111**:2023–2028. https://doi.org/10.1073/pnas.1316183111.